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Methimazole, thyroid hormone replacement, and lipogenic enzyme gene expression in broilers☆

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Abstract

The purpose of this experiment was to determine the possible relationship between certain indices of lipid metabolism and specific gene expression in chickens fed methimazole to simulate hypothyroidism. Male broiler chickens (*Gallus gallus*) growing from 7 to 28 days of age were fed diets containing 18% crude protein and either 0 or 1 g methimazole per kilogram of diet. At 28 days, these two groups were further subdivided into groups receiving 18% crude protein diets containing either 0 or 1 mg triiodothyronine (T_3) per kilogram. Birds were sampled at 28, 30, and 33 days. Measurements taken included in vitro lipogenesis (IVL), malic enzyme (ME) activity, isocitrate dehydrogenase, aspartate amino transferase, and the expression of the genes for ME, fatty acid synthase (FAS), and acetyl coenzyme carboxylase (ACC). Hypothyroidism decreased IVL and ME at 28 days of age; however, T_3 supplementation for 2 days restored both IVL and ME. Paradoxically, continuing T_3 replenishment for an additional 3 days decreased IVL but did not decrease ME activity. In contrast, supplemental T_3 decreased IVL in euthyroid birds, regardless of the dosing interval, but had no effect on ME activity. Although methimazole decreased ME gene expression, there was only a transitory relationship between enzyme activity and gene expression when plasma T_3 was restored with exogenous T_3. These data may help to explain some of the apparent reported dichotomies in lipid metabolism elicited by changes in the thyroid state of animals. In addition, most metabolic changes in response to feeding T_3 occurred within 2 to 5 days, suggesting that changes in intermediary metabolism preceded morphological changes. In conclusion, the thyroid state of the animal will determine responses to exogenous T_3.
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Keywords: Thyroid; Gene expression; Lipogenesis; Enzyme activities; Metabolism

1. Introduction

Although the thyroid gland partially controls avian growth, artificial changes in thyroid hormone levels do not always change growth predictably. In one study (Leung et al., 1985), dietary triiodothyronine (T_3) and thyroxine (T_4) decreased body weight and feed efficiency of chickens. In a previous study (Leung et al., 1984a,b), daily injections of thyroid-releasing hormone (TRH) improved growth and increased plasma thyroid hormone concentrations. In contrast, feeding T_3 increased plasma T_3 but failed to improve the growth weight of dwarf chickens (Leung et al., 1984a,b). Cogburn et al. (1989) also reported that dietary TRH increased plasma growth hormone (GH), thyroid hormone levels, and body weight. On the other hand, long-term, dietary administration of thyroid hormones in another study decreased both growth and fat deposition, with T_3 being more effective than T_4 (Decuypere et al., 1987). Other sets of data also suggested that dietary T_3 decreased body fat (Leung et al., 1984a,b), as well as plasma GH concentrations (Harvey, 1983). It should also be noted that chemical hypothyroidism, caused by either propylthiouracil

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(PTU) or methimazole, also decreased growth (Leung et al., 1985; Chiasson et al., 1979).

What is lacking from these reports is any information concerning birds' recovery from thyroid hormone perturbations (inhibition of T_3 production by feeding methimazole). Recently, we have proposed and tested the hypothesis that feeding T_3 after a 3-week methimazole challenge would rapidly restore circulating levels of T_3. Methimazole (1-methyl-2-mercaptimidazole), used to induce hypothyroid status in the present experiments, inhibits thyroidal production of thyroid hormones, but does not directly affect extrathyroidal 5′ deiodination of T_4 (Chopra et al., 1982).

Malic enzyme (ME) activity was monitored because of its role in providing reducing equivalents (NADPH) for the synthesis of fatty acids. Isocitrate: NADP+ oxidoreductase-[decarboxylating] (ICD) may function as both a residual source for the provision of NADPH and to provide a coreactant for transamination. Aspartate aminotransferase (AAT) aids in the removal of excess amine groups formed by feeding high-protein diets.

The purposes of this experiment were to further study the metabolic effects of hypothyroidism and repletion of plasma thyroid hormones, and to determine if changes the levels of mRNA for certain lipogenic enzymes related to changes in metabolic rates noted with thyroid states. We chose to analyze malic enzyme activity and gene expression because of its central role in providing reducing equivalents to support de novo lipogenesis. In our experience, measurements of in vitro lipogenesis (IVL) approximate the rate limiting activities of both acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS; Rosebrough and Steele, 1987).

2. Materials and methods

2.1. Animals—general

All chickens were held under a quarantine that was certified by the station veterinarian. Chickens were observed daily for healthiness. One authorized animal caretaker was assigned to maintain chickens over the course of the experiments. In addition, the research protocols were approved by the Beltsville Agricultural Research Animal Care Committee. Birds were allowed to consume both feed and water on an ad libitum basis. Birds were kept in electrically heated battery brooders (four birds/pen replicate) in an environmentally controlled room (22 C). A 12-h light (0600 to 1800 h), 12-h dark (1800 to 0600 h) cycle was maintained. The birds were killed by decapitation at 0900 h to minimize possible diurnal variation.

At 7 days of age, male broiler chickens were assigned to one of two dietary treatments [18% crude protein+0 or 1 g methimazole (METH)/kg diet) for a 7- to 28-day growth trial. At 28 days, the chickens were given a diet containing 18% crude protein+0 or 1 mg T_3 /kg to result in four treatment groups (18-18, 18-T_3, METH-18, METH-T_3). The first letter denoted the treatment from 7 to 28 days, and the second letter the treatment from 28 to 33 days. These dietary treatments formed a factorial arrangement with six pen replicates for each dietary treatment. The chickens were housed in battery brooders in an environmentally controlled room maintained at 20–23 C with a 12-h light–dark cycle (0600–1800 h light). At 0800 h, one chicken was then randomly selected from each pen replicate at 28, 30, and 33 days, weighed, and killed by decapitation. The livers were rapidly removed, sectioned into either a PBS and washed to remove blood and debris, or snap-frozen in liquid N_2.

2.2. In vitro techniques–in vitro Metabolism—Lipogenesis

Livers were then sliced (MacIlwain Tissue Chopper; 0.4–0.5 mm), and quadruplicate explants were incubated at 37 °C for 2 h in Hanks' balanced salts containing (Hanks and Wallace, 1949) 10 mM HEPES and 10 mM sodium[2-^{14}C]acetate (166 MBq/mol). All incubations were conducted in 3-mL volumes at 37 °C for 2 h under a 95% O_2–5% CO_2 atmosphere (Rosebrough and Steele, 1985; Rosebrough et al., 1988). At the end of the stated incubation periods, the explants were placed in 10 mL of 2:1 chloroform/methanol for 18 h according to Folch et al. (1957). The extracts were evaporated to dryness and dispersed in scintillation fluid. Radioactivity in the extracts was measured by liquid scintillation spectroscopy. In vitro lipogenesis was expressed as micromoles of acetate incorporated into lipids per gram of tissue.

2.3. In vitro metabolism—enzyme assay

Remaining liver tissues were homogenizeed (1:10, w/v) in 100 mM HEPES (pH 7.5) 3.3 mM β-mercaptoethanol and centrifuged at 12,000×g for 30 min (Rosebrough and Steele, 1985). The supernatant fractions were kept at −80 °C until analyzed for the activities of malate: NADP+ oxidoreductase-[decarboxylating] (malic enzyme—ME, EC 1.1.1.40), isocitrate: NADP+ oxidoreductase-[decarboxylating] (ICD-NADP, EC 1.1.1.42), and aspartate aminotransferase (AAT, EC 2.6.1.1).

Malic enzyme activity was determined by a modification of the method of Hsu and Lardy (1969). Reactions contained 50 mM HEPES (pH 7.5), 1 mM NADP, 10 mM $MgCl_2$, and the substrate, 2.2 mM L-malate (disodium salt) in a total volume of 1 mL. Portions (50 μL) of the 12,000×g supernatants (diluted 1:10) were preincubated in the presence of the first three ingredients. Reactions were initiated by adding the substrate and following the rate of reduction of NADP at 340 nm at 30 °C.

2.3.1. Isocitrate

NADP+ oxidoreductase-[decarboxylating] activity was determined by a modification of the method of Cleland et al. (1969). Reactions contained 50 mM HEPES (pH 7.5), 1 mM

NADP, 10 mM $MgCl_2$, and the substrate, 4.4 mM DL-isocitrate in a total volume of 1 mL. Portions (50 μL) of the 12,000×*g* supernatants (diluted 1:10) were preincubated in the presence of the first three ingredients. Reactions were initiated by adding the substrate and following the rate of reduction of NADP at 340 nm at 30 °C.

Aspartate aminotransferase activity was determined by a modification of the method of Martin and Herbein (1976). Reactions contained 50 mM HEPES, 200 mM L-aspartate, 0.2 mM NADH, 1000 units/L malate: NAD+ oxidoreductase (EC 1.1.1.37), and the substrate, 15 mM 2-oxoglutarate in a total volume of 1.025 mL. Portions (25 μL) of the 12,000×*g* supernatants (diluted 1:20) were preincubated in the presence of the first four ingredients. Reactions were initiated by adding the substrate and following the rate of oxidation of NADH at 340 nm at 30 °C. Enzyme activities are expressed as micromoles of product formed per minute under the assay conditions (Rosebrough and Steele, 1985).

2.4. Lipogenic enzyme gene expression

Reverse Transcription Polymerase Chain Reaction (RT-PCR): total RNA was isolated using the Tri-Reagent procedure (Life Technologies, Rockville, MD) and quantified spectrophotometrically. Optical density readings were expressed as ratios of 260/280 nm. Ratios less than 1.9 were considered as indicative of low-quality RNA. In addition, extracted RNA quality was evaluated by agarose gel electrophoresis. RT reactions (50 μL) consisted of 5 μg total RNA, 100 units MMLV reverse transcriptase (RNase H minus, point mutation), 40 units RNAsin, 1.0 mM of each dNTP, and 6 pmol random hexamer primers (Promega, Madison, WI). Hot-started PCR was performed in separate 27.5 μL reactions containing PCR buffer, 0.5 (FAS, ACC) or 1.25 (ME) units of Platinum Taq DNA polymerase (Life Technologies), 0.2 mM of each dNTP, 10 pmol each of each gene-specific primer including a set for β-actin (see below), the internal standard. Each PCR was run as a duplex with primer sets added for a particular lipogenic enzyme and for β-actin. The final concentration of magnesium in the reaction was either 1.4 mM (FAS, ACC) or 1.8 mM (ME). PCR thermal cycling parameters were as follows: 1 cycle 94 °C for 2 min, followed by 30 cycles, 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min with a final extension at 72 °C for 8 min. RT-PCR produced dsDNA amplicons of 423, 431, 447, and 300 bp for FAS, ME, ACC, and β-actin, respectively.

2.4.1. Gene-specific primers

The following gene-specific primer sets were used:

Gene	Sense primer	Antisense primer
Fatty acid synthase	5′-AGGAGATTCCAGCATCGTGCAGC	5′-GGAGTCAAACTAGTTATCCATGGCC
Malic enzyme	5′-TGAAGAGGGGCTACGAGGT	5′-CCCATTCCATAACAGCCAAG
Acetyl-CoA carboxylase	5′-CACTTCGAGGCGAAAAACTC	5′-GGAGCAAATCCATGACCACT

2.4.2. Capillary electrophoresis (CE)

Aliquots (2 μL) of RT-PCR samples were diluted 1:100 with deionized water prior to analysis by CE. A P/ACE MDQ (Beckman Coulter, Fullerton, CA), configured for reversed polarity and equipped with an argon ion laser-induced fluorescence (LIF) detector, was used to separate and detect the dsDNA amplicons. A μSIL-DNA capillary (J and W Scientific, Folsom, CA) with a 75-μm I.D., 0.075-μm film thickness, and length of 32 cm was used at 25 °C. The dsDNA separation buffer was from Sigma (St. Louis, MO). EnhanCE™ dye (1 mg/mL stock in methanol) was added to the separation buffer to produce a final concentration of 0.5 μg/mL. Diluted RT-PCR samples were loaded by electrokinetic injection at 3.5 kV for 5–10 s. Separations were performed at a field strength of 300 V/cm (8.1 kV) for 4.5 min.

2.4.3. Data analysis

P/ACE MDQ software (Beckman Coulter) was used to calculate integrated peak areas. A lipogenic enzyme/β-actin peak area ratio was then calculated and used to compare tissue samples with respect to relative lipogenic enzyme gene expression activities. Ratio values are expressed as mean±S.E.M.

2.5. Statistical procedures

Significance of the two treatments (±methimazole) at 28 days was determined with a *t*-test. From this time on, the experiment was considered as a 2×2×3 factorial arrangement. The main treatments were pre- and post-28-day thyroid status and time following initiation of the T_3 supplementation.

3. Results

Table 1 summarizes methimazole effects on broiler chickens growing from 7 to 28 days. Chickens fed methimazole were lighter and ate less food than controls ($P<0.01$). Although birds fed methimazole were smaller, their livers were larger ($P<0.01$) and, therefore, the relative liver size was over twice that of controls ($P<0.01$). In contrast, relative fat pad sizes (gram abdominal fat per kilogram of body mass) were similar for both treatment groups.

Table 2 summarizes methimazole effects of some indices of intermediary metabolism in broilers. Methimazole

Table 1
Dietary methimazole (METH) effects on broilers growing from 7 to 28 days[a]

Diet	28-day mass (g)	Feed intake (g)	Liver weight (g)	RLS[b] (g/kg mass)	AFP[c] (g)	RFPS[d] (g/kg mass)
18	962±38.4	1629±37.2	19.8±1.2	20.6±1.1	8.6±1.0	0.89±0.07
METH	535±23.7[e]	788±15.7[e]	25.6±1.7[e]	47.8±1.3[e]	5.2±0.7[e]	0.97±0.05

[a] Broilers growing from 7 to 28 days of age were fed diets containing 18% protein and either 0 or 1 g of methimazole (METH) per kilogram of diet.
[b] Relative liver size—gram liver per kilogram of body mass.
[c] Abdominal fat pad.
[d] Relative fat pad size—gram fat pad per kilogram of body mass.
[e] Significant effect of methimazole.

decreased both IVL and ME but increased AAT activities in 28-day-old birds. This table also summarizes the effect of methimazole on subsequent responses to supplemental T_3. Supplemental T_3, fed to birds given the control diet from 7 to 28 days (18-T_3), decreased IVL 2 days following the onset of the T_3 supplementation. Two days of T_3 supplementation increased lipogenesis in birds previously given methimazole (METH-T_3), but decreased IVL after an additional 3 days of supplementation. In contrast, refeeding the 28-day methimazole group with a normal, unsupplemented diet (METH-18) increased IVL at 30 days with a further increase occurring at 33 days. In a fashion similar to that noted with IVL, methimazole decreased ME activity in the 28-day-old bird. With the exception of METH-T_3 (METH-T_3>METH-18) at 30 days, T_3 supplementation had little effect on ME activity.

Table 3 summarizes treatment effects on the expression of genes controlling the activity of the above enzymes. Feeding METH decreased the apparent expression of all three genes at 28 days. Refeeding the METH group with a diet containing T_3 (METH-T_3) increased (P<0.05) ME gene expression at 30 days compared to the other three treatment groups (18-18, 18-T_3, and METH-18). In contrast, this difference in ME gene expression was not seen at 33 days. Feeding T_3 increased (P<0.05) ACC gene expression at 30 days in birds fed METH for the 7- to 28-day growth period. In contrast, dietary T_3 decreased ACC gene expression in birds fed the control diet for the initial period. The effects of T_3 on ACC gene expression in both groups persisted at day 33. Dietary T_3 increased (P<0.05) FAS gene expression on day 30 in birds made fed METH for days 7 to 28. In contrast, this effect was not present in this treatment group on day 33. Dietary T_3 decreased FAS gene expression in those birds fed the control diet from day 7 to day 28.

4. Discussion

A partial relationship was established between malic enzyme gene expression, enzyme activity, and in vitro lipogenesis. This report shows that manipulation of plasma thyroid hormone levels with methimazole decreases lipogenic gene expression and malic enzyme activity. It was noted that initial repletion of plasma T_3 was accompanied by an increase in malic enzyme gene expression, an increase in enzyme activity, and an increase in lipogenesis. These data do suggest some degree of disconnect between gene expression and enzyme activity, however, especially after plasma T_3 restoration. These data present an interesting contrast to a previous study of ours (Richards et al., 2003) that demonstrated a close correlation between lipogenic gene expression and concomitant enzyme activities. In

Table 2
Dietary methimazole (METH) effects on in vitro metabolism in broilers growing from 7 to 28 days[a,b]

Days	Treatment	ME/g	ICD/g	AAT/g	IVL/g
Day 28	18	21.7±1.1	28.7±2.6	70.9±2.4	37.2±1.1
Day 28	METH	1.9±0.5[c]	26.4±1.8	126.6±20.7[c]	7.2±1.5[c]
Day 30	18-18	16.7±2.5	29.9±0.8	68.5±1.7	35.3±6.1
Day 30	18-T_3	17.9±1.7	37.4±2.2	89.6±2.1[d]	12.8±1.4[d]
Day 30	METH-18	3.8±0.5	31.6±3.0	85.5±5.4	19.0±2.1[d]
Day 30	METH-T_3	7.9±0.6[d]	42.2±1.7[d]	201.3±24.1[d]	30.5±2.1[d]
Day 33	18-18	15.5±1.7	26.8±2.3	61.3±7.6	26.9±2.4
Day 33	18-T_3	11.8±1.1[d]	33.8±1.8	73.7±3.4	9.5±1.0[d]
Day 33	METH-18	9.9±1.0[d]	34.3±3.0	94.4±5.5	25.8±1.2[d]
Day 33	METH-T_3	11.0±1.2[d]	36.0±2.6	119.6±9.0	15.5±1.1[d]

[a] Broilers growing from 7 to 28 days of age were fed diets containing 18% protein and either 0 or 1 g of methimazole (METH) per kilogram of diet. At 28 days, birds were switched to diets containing the same level of protein and either 0 or 1 mg T_3/kg. The first expression in each legend denotes the dietary treatment for the 7- to 28-day period. The second expression denotes the treatment for 28 to 33 days. All treatment groups were sampled at 28, 30, and 33 days.
[b] ME, malic enzyme; ICD, NADP-isocitrate dehydrogenase; AAT, aspartate aminotransferase; IVL, in vitro lipogenesis. Enzyme activities are expressed as micromole of reduced or oxidized NAD(P) formed per minute under standard assay conditions.
[c] Significant effect of methimazole at 28 days.
[d] Significant difference between a value at either day 30 or 33 and its corresponding treatment at value at 28 days (i.e., METH-18 vs. METH).

Table 3
Dietary methimazole (METH) effects on the expression of certain genes implicated in lipid metabolism in broilers growing from 7 to 28 days[a,b]

Days	Treatment	ME	ACC	FAS
Day 28	18	0.39±0.13	1.07±0.02	1.09±0.11
Day 28	METH	0.18±0.06[c]	0.76±0.02[c]	0.79±0.03[c]
Day 30	18-18	0.40±0.02	0.94±0.03	1.03±0.01
Day 30	18-T_3	0.47±0.03	0.67±0.04[d]	0.81±0.07
Day 30	METH-18	0.45±0.03[d]	0.69±0.04	0.71±0.04
Day 30	METH-T_3	0.78±0.07d[d]	1.04±0.06[d]	1.27±0.06[d]
Day 33	18-18	0.55±0.05	0.97±0.05	0.94±0.04
Day 33	18-T_3	0.44±0.05	0.55±0.07[d]	0.59±0.08[d]
Day 33	METH-18	0.52±0.01[d]	0.92±0.03	0.67±0.00
Day 33	METH-T_3	0.47±0.03[d]	0.78±0.04	0.62±0.02

[a] Broilers growing from 7 to 28 days of age were fed diets containing 18% protein and either 0 or 1 g of methimazole (METH) per kilogram of diet. At 28 days, birds were switched to diets containing the same level of protein and either 0 or 1 mg T_3/kg. The first expression in each legend denotes the dietary treatment for the 7- to 28-day period. The second expression denotes the treatment for 28 to 33 days. All treatment groups were sampled at 28, 30, and 33 days.

[b] Gene expression is noted as the ratio of the expression of the desired gene: β actin gene expression. ME, malic enzyme; ACC, acetyl coenzymeA carboxylase; FAS, fatty acid synthase.

[c] Significant effect of methimazole at 28 days.

[d] Significant difference between a value at either day 30 or 33 and its corresponding treatment at value at 28 days (i.e., METH-18 vs. METH).

fairness, the latter study was conducted with normal, euthyroid birds undergoing light stimulation to induce egg production. This study shows that supplemental T_3 enhanced lipogenesis in the hypothyroid chicken, but that enhancement only lasted until restoration of normal levels of plasma T_3 occurred. At this point, it should be noted that T_3 supplementation did not restore lipogenesis to control levels, although plasma T_3 concentrations were much greater than controls. At some point, thyroid function in the hypothyroid bird appears to have been restored by exogenous T_3, and further T_3 supplementation decreased lipogenesis like that noted in the euthyroid bird.

Malic enzyme, along with other enzymes controlling lipogenesis, responds to both hypo- and hyperthyroid conditions. Thus, malic enzyme gene expression and activity are another useful tool in assessing thyroid function in the bird. In contrast to the rat, however, T_3 administration did not increase ME activity 10-fold over that noted in the hypothyroid rat. It is also important to realize that although ME may provide the necessary NADPH for lipogenesis, the enzyme may not strictly regulate lipogenesis according to the data in this study. A more plausible explanation is that ME reflects NADPH utilization and may not regulate fatty acid synthesis.

Sood et al. (1996) noted that ME mRNA levels in liver followed a pattern of expression similar to that of the ME activity levels. They did show the existence of isoforms of ME mRNA (27S and 21S) with the 27S in much greater quantity. Dozin et al. (1986) indicate that both isoforms coded for ME protein. This finding seemed to support previous work by Strait et al. (1989) that also showed the existence of these two mRNA species. The latter work did indicate that the level of ME activity was related to both species. Strait et al. (1989) previously sequenced the 27S species and determined that polyadenylation was responsible for this species size and its sensitivity to T_3 administration. They also proposed that a delayed response time in the expression of the 21S fragment was due to a defective polyadenylation site. Thus, message processing may be a further point of regulation of both message stability and translation efficiency.

Swierczynski et al. (1991) found that the addition of triiodothyronine (T_3) to chick–embryo hepatocytes in culture increased accumulations of ME, FAS, ACC, and their mRNAs. Hillgartner et al. (1997) reported that (1) T_3 enhanced ACC gene expression and (2) glucose acted in consort to stimulate enhanced gene expression. Goodridge et al. (1998) described T_3 response units in the 5′-flanking region of the ME gene. Although this region contained only one major response unit, several minor units were also noted. These minor units, however, were necessary for full gene expression. It is unclear from our data if occupancy of these response units could play a role in gene expression after the initial repletion interval in this study.

As previously stated, the results of this study may present some conflict with the possible nexus between gene expression and enzyme activity. A link was noted in 28-day-old birds fed either control or methimazole-containing diets. Methimazole, used to induce hypothyroidism, decreased ME gene expression and enzyme activity. In contrast, although restoring plasma T_3 increased both gene expression and enzyme activity 2 days postrestoration, it should be noted that ME activity continued to increase over the entire 5-day experimental period without a corresponding increase in gene expression. It seems evident that changes in gene expression may control enzyme activity during challenges that radically alter metabolism (fasting–refeeding, high–low protein diets, initial stages of hormone oblation–replenishment), but message stability and enzyme protein turnover may control activity as homeostasis is reached.

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